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Tissue and Nerve Growth Promoting Factors

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Biological Aspects of Specific Growth Promoting Factors

The recognition of the growth promoting activity elicited by a variety of agents belonging to the class of hormones as well as to the less well defined category of 'growth factors' dates back to the second half of the past century. However, only much more recently have the growth effects elicited by various agents been submitted to a rigorous analysis and attempts made to elucidate how they act on the target cells. All these investigations centred mainly if not exclusively on the study of hormones: the ill-defined class of 'growth factors' was only occasionally the object of analysis. In these few instances the study was restricted to the exploration of the effects elicited by given growth agents on cell populations cultured in liquid or semi-solid media.

This paper discusses two growth factors which were recently characterized and assayed *in vivo* and *in vitro*. Both factors markedly enhance the growth of given cell types leaving all other cells unaffected; both factors play an outstanding role in the life of the target cells. Similar control mechanisms may exist for all cell types and this possibility raises the question of the biological significance of 'specific growth factors' and of their relationship to hormones.

The Nerve Growth Factor

In 1952 we reported on the discovery of a diffusible nerve growth factor released by mouse sarcomas 180 and 37 (Levi-Montalcini 1952). This report was based on a long series of studies of the effects elicited by these tumours in the chick embryo (Levi-Montalcini & Hamburger 1951). When implanted into the body wall of 3-day chick embryos, or on to the chorioallantoic membrane of 4–6-day chick embryos, both tumours cause striking developmental changes in the sympathetic para- and pre-vertebral ganglia. The ganglia markedly increase in size, due to an increase in cell number and cell size of the sympathetic neurones. The ganglia reach a volume four to six times larger than control ganglia and produce an excessive number of nerve fibres which enter in large bundles into most of the embryonic viscera. They also force their way into the lumen of large and small blood vessels. The density of these fibres in some organs like the mesonephros (which normally is not innervated) is such as to disrupt the structural and functional organization of the organ.

A less striking but marked increase in size is also apparent in sensory ganglia of embryos bearing intracœlomatic transplants of the same tumours. Unlike the sympathetic ganglia, the sensory ganglia are receptive to the growth promoting effects of mouse sarcomas only during a restricted period of their early differentiation. In chick embryos this period occurs between the sixth and the twelfth day of incubation (Levi-Montalcini 1963).

The effect of the tumour growth factor was then investigated in sensory and sympathetic ganglia cultured *in vitro* by the hanging drop technique. It was shown that in proximity to a fragment of the neoplastic tissue both types of ganglia produce a dense halo of nerve fibres in eight to twelve hours, while no fibres grow from control ganglia in the same period (Levi-Montalcini *et al.* 1954). The growth factor present in these mouse sarcomas was identified in a heat-labile protein particle (Cohen *et al.* 1954).

While these studies were in progress, two much more potent sources of the same or of closely related nerve growth factors were discovered in snake venom (Cohen & Levi-Montalcini 1956) and in mouse salivary glands (Levi-Montalcini & Cohen 1960). The assumption that these newly discovered nerve growth factors are very similar if not identical with the neoplastic nerve growth factors was based on extensive biochemical investigations. When injected into the yolk of developing chick embryos, both factors elicit the same growth response from sympathetic ganglia as the implanted mouse sarcomas. Their *in vitro* effects on sensory and sympathetic ganglia are identical with the effects elicited by a fragment of mouse sarcoma or cell-free extract of the mouse tumours (Levi-Montalcini 1958). The biochemical analysis of the venom and salivary factors indicated that in both instances the active agent was a protein (Cohen 1958).

The nerve growth factor (NGF) was further purified from mouse salivary glands, where it is present in a concentration about six thousand times higher than in mouse tumours, and was then used in all subsequent investigations.

The purest preparation of the salivary NGF, which was identified as a single protein component with a sedimentation constant of 4·3S. (Cohen 1960) was then assayed in newborn mice. Daily injections of this fraction for nine days result in the production of para- and pre-vertebral sympathetic ganglia ten to twelve times larger than control ganglia. This remarkable growth effect is due to increased mitotic activity as well as to an increase in the size of individual neurones. These cells grow much larger than fully differentiated neurones of adult mice. In the adult, the NGF elicits increase in size of sympathetic nerve cells but no increase in their number (Levi-Montalcini & Booker 1960*a*). The marked hypertrophy and hyperplasia of sympathetic ganglia of treated animals result in turn in the hyperinnervation of the viscera which does not appear to interfere with the normal function of these organs. The treated animals in fact develop as well as controls.

While these results gave evidence of the striking growth response called forth by the NGF in sympathetic nerve cells of embryonic, newborn and adult organisms, the following observations lead us to consider the NGF as essential to the very life of the responsive nerve cells.

In 1959 Cohen produced an antiserum to the NGF by injecting the purified growth factor with Freund adjuvant into the pads of rabbits (Cohen 1960). The injection of minute amounts of this antiserum in newborn mammals immediately blocks the growth and differentiation of developing sympathetic nerve cells and kills already differentiated neurones. As a result, the sympathetic ganglia undergo almost total atrophy (Levi-Montalcini & Booker 1960*b*). The disappearance of 95–98% of the whole sympathetic cell population of para- and pre-vertebral ganglia was verified by histological analysis of the treated animals one to three years after the injection of the antiserum. No adverse effects are apparent in other tissues or organs. The dramatic and almost instantaneous destruction of the sympathetic cell population upon injection of the antiserum to the NGF indicates that these cells cannot survive in the absence of this growth factor. Additional evidence in support of the hypothesis that the NGF plays a fundamental role in the life of sympathetic nerve cells came from experiments performed *in vitro* on ganglia treated with trypsin. Under these conditions, individual nerve cells survive and develop in liquid synthetic media enriched with NGF at a molar concentration of 10^{-6}. In its absence all nerve cells rapidly disintegrate and die (Levi-Montalcini & Angeletti 1963).

Thus we came to the conclusion that the NGF plays a role in the life of the sympathetic nerve cells which has no parallel in other well known growth regulators such as nutrients, vitamins and hormones. The NGF acts in extremely low concentration, is highly selective and specific in its effects, is normally present in the receptive nerve cells (Levi-Montalcini & Angeletti 1961) and moreover proves to be essential to their subsistence, at least during an early phase of their differentiation.

The mechanism whereby the NGF calls forth this remarkable response from the receptive nerve cells was recently the object of a series of metabolic studies (Toschi *et al.* 1964, Angeletti *et al.* 1964, Levi-Montalcini & Angeletti 1965). Evidence was presented that the NGF markedly stimulates the rate of amino-acid incorporation and turnover in embryonic sensory nerve cells *in vitro* and in embryonic and mature sympathetic nerve cells *in vivo* and *in vitro*. Under these conditions, the NGF calls forth an even higher increase in RNA synthesis as proved by experiments with labelled uridine. Experiments with the inhibitors puromycin and actinomycin-D suggest that the stimulation of protein synthesis by NGF depends on a primary effect on nuclear RNA synthesis.

The Epidermal Growth Factor
While the analysis of the NGF was in progress and attempts were being made to elucidate its mechanism of action, another equally potent and

highly specific growth factor was discovered in the same mouse submaxillary salivary glands by Stanley Cohen. This factor, designated from its activity as the epidermal growth factor (EGF), is present in high concentration in the tubular portion of the submaxillary salivary glands of the adult male mouse (Cohen 1962). It is in this part of the gland that the NGF was also detected. The two growth factors are in fact so closely linked to each other that they can be separated only by extensive purification procedures. It was the presence of some side-effects on the skin elicited by crude NGF which gave Stanley Cohen the lead for the discovery and further purification of EGF.

EGF is a protein. Cohen has calculated that its molecular weight is of the order of 14,000. It is heat-stable, not dialysable, destroyed by incubation with proteolytic enzymes and inactivated by a specific antiserum. Two amino acids, phenylalanine and lysine are lacking. Their absence in the purified fraction is taken as the criterion of purity since 'it might be expected that contaminating polypeptides would contain these amino acids' (Cohen 1964).

EGF has a remarkable growth effect on epithelial structures. When daily injections of 0·5 μg per 1·5 g of body weight are given to newborn mice for six days, a marked increase occurs in thickness and precocious keratinization of the skin as well as an increase in height of the epithelium lining the oral cavity, the œsophagus and the stomach mucosa. *In vitro*, EGF elicits a striking growth effect on fragments of embryonic chick skin. While control epithelial tissues, cultured in liquid synthetic media, remain unchanged for the first forty-eight hours of culture, comparable skin fragments cultured in the presence of EGF at the concentration of 0·1 μg/ml proliferate markedly. Over longer periods of culture, EGF stimulates the subsequent keratinization of the epidermal cells.

Production and Biological Significance of the Growth Factors

As mentioned above, two highly specific and potent growth factors were isolated from the tubular portion of the adult mouse submaxillary salivary glands, and their properties were assayed in living organisms as well as *in vitro*. At present, experiments are in progress to search for other possible growth promoting activities of the salivary gland extract on other cell types beside the nerve cells and the epithelial cells.

As for the production of the two growth factors isolated in large quantities from the above source, two alternative possibilities were considered: either the two factors are synthesized and released from the salivary gland in the same way as hormones are produced by endocrine glands, or they are produced elsewhere in the organism and are released through the salivary gland excretory channels. The second alternative seems likely (Levi-Montalcini & Angeletti 1960, Levi-Montalcini 1962). The finding that NGF is present also in mouse sarcomas and in granuloma tissue (Levi-Montalcini & Angeletti 1960) suggested the possibility that this growth promoting factor might be produced in the organism everywhere by cells of mesenchymal type and be utilized by nerve cells of the type described above. If this hypothesis turned out to be correct, one would still be faced with many unsolved problems such as its presence in large quantity in the mouse salivary gland and in the snake venom gland and its absence from the salivary glands of other vertebrates. Also it remains to be seen whether other specific growth factors such as those described above exist and, if so, whether they bear any relationship with the hormones.

This last question is not only a semantic one. Indeed our hesitation in defining the category of the growth factors reflects our ignorance of the precise role of these agents which seem to share some properties with hormones but are unlike them in many important respects. A hormone, as defined by the Shorter Oxford dictionary, is a 'substance formed in an organ and serving to excite some vital process'. These substances, as is well known, are the product of specialized endocrine glands.

The two specific growth factors, NGF and EGF do not fit this concept of a hormone. There is no indication that they are produced in a well-defined organ or organs. Their primary effect is to stimulate the growth of the responsive cells rather than to stimulate their functional activity. It was the excessive proliferation and overgrowth of the sympathetic nerve cells which impressed us from the beginning of this investigation. The massive invasion of embryonic organs by sympathetic nerve fibres as well as the penetration of these fibres into the blood vessels, while stressing the magnitude of the growth response, also indicated that this excessive growth of the sympathetic system was detrimental rather than beneficial to the organism as a whole.

That NGF plays a most important, and we should say essential, role in the life of the target cells is shown by the dramatic effects elicited by the antiserum to NGF and by the demonstration that isolated nerve cells can only survive and grow in the presence of NGF. This cannot be said of hormones which stimulate the receptive organs or tissues but are not indispensable for their survival and growth.

A last but not less important difference between hormones and our growth factors is temporal in nature. Hormones display their function rather late in life. Although hormonal effects are already apparent during fœtal life, it is in the post-natal life and in the fully grown organisms that the role of hormones becomes prominent. Our growth factors on the contrary are most important during early growth and differentiation of the target cells. Indeed some of these cells, such as the sensory nerve cells, are receptive to the growth effect of the NGF only during a very restricted and early period of their growth. Even the sympathetic nerve cells, which respond to this agent throughout life, show a maximal growth response during the early phase of their differentiation. The same is true for the epidermal growth factor.

It is tempting to suggest that specific growth factors such as those described might be regarded as a sort of more primitive and fundamental integrative system than hormones. They are possibly metabolites released by cells still not organized in well defined organs and utilized by other cells as growth factors. Since their main function is indeed to promote growth in the responsive cells, the non-committal term of 'growth factors' seems to be appropriate at present, though we should be ready to replace it with a more precise term as our knowledge of these remarkable biological agents gains in precision and depth.

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Size, Growth, and Growth-control in Developing Animals

Introduction
The quite extraordinary phenomenon discovered by Levi-Montalcini & Hamburger (1951) and so successfully explored since by Levi-Montalcini and her colleagues will clearly have to be accommodated within any general theory of growth control. This will be so even if nerve growth factor (NGF) itself proves to have a restricted role in determining normal growth patterns, because the specific effects of anti-NGF serum must surely mean that NGF is an essential, if not necessarily a limiting, factor in normal development. It may therefore be worth referring briefly to some of the more general problems of growth control that developmental biology has so far tackled.

It is, of course, almost axiomatic that whole organisms and many of their parts are capable of growing at rates far in excess of those actually achieved. The decline of specific growth rate with age is not, as Medawar has pointed out, a process with an in-built inevitability to be compared with, say, the increase of entropy in an open system. Indeed, there are many circumstances in which it can be temporarily or locally overcome, as in the catch-up growth of individuals restored to favour after a period of ill-health or malnutrition (*see* e.g. Tanner 1964) or in the proliferation released by the conditions of *in vitro* culture. NGF, in this sense, is not alone. Even those organs which are not demonstrably growing at all, but which do have a low rate of cellular replacement, may have this rate lowered by